

LIGNIN BIOSYNTHESIS

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■ **Abstract** The lignin biosynthetic pathway has been studied for more than a century but has undergone major revisions over the past decade. Significant progress has been made in cloning new genes by genetic and combined bioinformatics and biochemistry approaches. In vitro enzymatic assays and detailed analyses of mutants and transgenic plants altered in the expression of lignin biosynthesis genes have provided a solid basis for redrawing the monolignol biosynthetic pathway, and structural analyses have shown that plant cell walls can tolerate large variations in lignin content and structure. In some cases, the potential value for agriculture of transgenic plants with modified lignin structure has been demonstrated. This review presents a current picture of monolignol biosynthesis, polymerization, and lignin structure.

CONTENTS

INTRODUCTION	520
LIGNIN COMPOSITION AND STRUCTURE	520
General Aspects	520
A Broader Definition of Lignins	522
VARIABILITY AND TOPOCHEMISTRY	523
MONOLIGNOL BIOSYNTHESIS	524
TRANSGENIC PLANTS	528
TRANSPORT OF MONOLIGNOLS	531
DEHYDROGENATION	532
POLYMERIZATION	533
Radical Generation and Radical Coupling	533
Polymerization Process	533
Nucleation Sites	534
BIOTECHNOLOGY	535
CONCLUSIONS AND PERSPECTIVES	536

INTRODUCTION

Lignin is, after cellulose, the second most abundant terrestrial biopolymer, accounting for approximately 30% of the organic carbon in the biosphere. The ability to synthesize lignin has been essential in the evolutionary adaptation of plants from an aquatic environment to land. Lignin is crucial for structural integrity of the cell wall and stiffness and strength of the stem (23, 71). In addition, lignin waterproofs the cell wall, enabling transport of water and solutes through the vascular system, and plays a role in protecting plants against pathogens (126).

Although researchers have studied lignin for more than a century, many aspects of its biosynthesis remain unresolved. The monolignol biosynthetic pathway has been redrawn many times and remains a matter of debate (36, 67). Likewise, the biochemical processes leading to dehydrogenation of the monolignols in the cell wall and their polymerization and deposition are fields of active discussion (31, 60, 81, 122, 128). In addition, we are only beginning to understand the transcriptional and posttranslational mechanisms and metabolic complexes regulating the flux through the phenylpropanoid and monolignol biosynthetic pathways.

LIGNIN COMPOSITION AND STRUCTURE

General Aspects

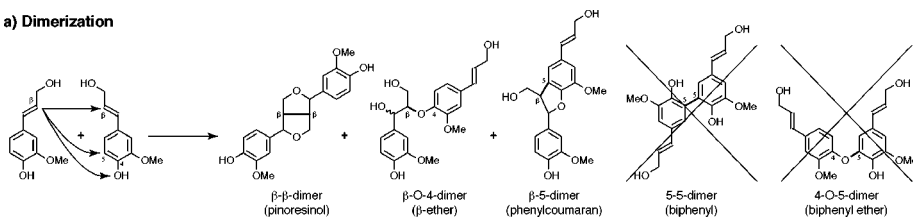
Lignins are complex racemic aromatic heteropolymers derived mainly from three hydroxycinnamyl alcohol monomers differing in their degree of methoxylation, *p*-coumaryl **M1H**, coniferyl **M1G**, and sinapyl **M1S** alcohols (50) (Figure 1) (a Supplemental Three-Dimensional View is available online: Follow the Supplemental Material link from the Annual Reviews home page at <http://www.annualreviews.org>). These monolignols produce, respectively, *p*-hydroxyphenyl **H**, guaiacyl **G**, and syringyl **S** phenylpropanoid units when incorporated into the lignin polymer. The amount and composition of lignins vary among taxa, cell types, and individual cell wall layers and are influenced by developmental and environmental cues (18). Although exceptions exist, dicotyledonous angiosperm (hardwood) lignins consist principally of **G** and **S** units and traces of **H** units, whereas gymnosperm (softwood) lignins are composed mostly of **G** units with low levels of **H** units. Lignins from grasses (monocots) incorporate **G** and **S** units at comparable levels, and more **H** units than dicots (11). **H** units, derived from the incorporation of the monolignol *p*-coumaryl alcohol **M1H** into lignins, should not be confused with *p*-coumarate esters **Y3**, which appear as pendant groups acylating grass lignins—this confusion (and the derivation of identical products using degradative methods such as nitrobenzene oxidation) has led to overestimation of **H** levels in the past.

Lignification is the process by which units are linked together via radical coupling reactions (50, 126). The main “end-wise” reaction couples a new monomer (usually a monolignol and usually at its β position) to the growing polymer, giving

rise to structures **A**, **B**, and **D2** (all of which are β -linked). Coupling between pre-formed lignin oligomers results in units linked 5–5 **D** and 5–O–4 **E**. The coupling of two monolignols is a minor event, with resinol (β – β) units **C** or cinnamyl alcohol end groups **X1** as the outcome. Monolignol dimerization and lignin are substantially different processes (2), explaining why lignification produces frequencies of the various units that are different from those produced by dimerization (Figure 2) or bulk polymerization *in vitro* (see below).

The interconnections are described in Figure 1. The most frequent inter-unit linkage is the β –O–4 (β -aryl ether) linkage **A**. It is also the one most easily cleaved chemically, providing a basis for industrial processes, such as chemical pulping, and several analytical methods. The other linkages are β –5 **B**, β – β **C**, 5–5 **D**, 5–O–4 **E**, and β –1 **F**, which are all more resistant to chemical degradation. The relative abundance of the different linkages depends largely on the relative contribution of a particular monomer to the polymerization process. For example, lignins composed mainly of **G** units, such as conifer lignins, contain more resistant

a) Dimerization



b) Lignification

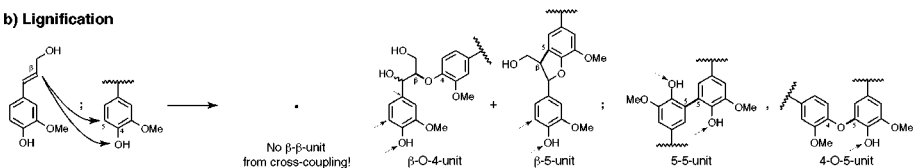


Figure 2 Lignification differs substantially from simple dimerization of monolignols. (a) Dimerization of coniferyl alcohol produces only three dimers, in each of which at least one of the coniferyl alcohols is coupled at its β position. The 5–5 and 5–O–4 dimers shown in most texts (and crossed out) do not actually arise in any significant way from monomer dimerization reactions. The new bond formed by the radical coupling reaction is noted in bold. (b) Cross-coupling of coniferyl alcohol with a **G** unit gives only two main products, explaining why there are more β -ethers formed during lignification than in monolignol dimerization (or in synthetic dehydrogenation polymer (DHP) synthesis, where dimerization is too frequent). Coupling of preformed oligomers is the source of most of the 5–5- and 5–O–4 units. Sites of further coupling reactions during lignification are indicated by dotted arrows. Not shown: Sinapyl alcohol analogously dimerizes to only two products (not the β –5 analog), and polymerization between a monolignol (either coniferyl or sinapyl alcohol) and an **S** unit in the polymer has only one outcome—the β –O–4 unit, explaining why high **S** lignins have elevated β -ether levels. Neither 5–5 nor 5–O–4 units can be formed between **S** units; cross-coupling of **G** and **S** units can furnish 5–O–4-linked structures.

(β -5 **B**, 5-5 **D**, and 5-O-4 **E**) linkages than lignins incorporating **S** units because of the availability of the C₅ position for coupling.

A Broader Definition of Lignins

It is becoming increasingly clear that lignins are derived from several more monomers than just the three monolignols **M1** (bold in Figure 1). Many “normal” plants contain lignins substantially derived from other monomers, and all lignins contain traces of units from apparently incomplete monolignol biosynthesis and other (side-)reactions that occur during that biosynthesis (113). Many of these units have been recently identified by their more substantial incorporation into lignins in transgenic and mutant plants with perturbations in the monolignol biosynthetic pathway.

Various acylated lignin units **Y** were suspected to derive from acylated monolignols **M9–M11** (95). *p*-coumarates **Y3** on grass lignins are regio-specifically attached to the γ position of lignin side-chains, and on all types of lignin units, suggesting that they are not products of postlignification derivatization (85). Recent identification of novel β - β -coupling products from kenaf lignins that could have only arisen from sinapyl acetate **M9S** (i.e., preacetylated sinapyl alcohol) provides more compelling evidence (86) and suggests that all of the acylated lignins (*p*-hydroxybenzoates **Y2** in poplars, palms, and willows; *p*-coumarates **Y3** in all grasses; and acetates **Y1** in palms and kenaf, as well as the low levels in many hardwoods) derive from acylated monolignols **M9–M11**. Because acetylated components can represent a significant part of the polymer (over 50% of kenaf bast fiber lignin units are acetylated, for example), they should be considered to be authentic lignin “monomers.”

Ferulates **M4G**, and their dehydrodimers that derive from radical coupling reactions and generate polysaccharide-polysaccharide cross-linking, are also intimately incorporated into lignins, particularly in grasses where they appear to function as nucleation sites for lignin polymerization (69, 109). Tyramine ferulate **M8G** (and possibly other hydroxycinnamate analogs) is intimately polymerized into the polymer in normal tobacco and is particularly enhanced in cinnamoyl coenzymeA (CoA) reductase (CCR)-deficient transgenic tobacco (110).

Dihydroconiferyl alcohol (DHCA) **X5G** and derived guaiacylpropane-1,3-diol units **X6G** are always detectable in gymnosperm lignins, suggesting that the monomer **M5G** is always produced with coniferyl alcohol **M1G** (111). DHCA-derived units **X5G** and **X6G** are major components of the lignin in a cinnamyl alcohol dehydrogenase (CAD)-deficient pine mutant where about half are involved in 5-5-coupled structures **D** (114). Similarly, cinnamyl **X2** and benzyl **X3** aldehyde groups are always detected in lignins. Whether they arise from postlignification oxidation reactions or from incorporation of the aldehyde monomers into the polymer is unclear, but the latter is highly implicated by the recent observations of increased levels in CAD-deficient plants, which also display products of end-wise hydroxycinnamyl aldehyde incorporation into the growing polymer—the hydroxycinnamyl aldehyde 8-O-4-linked units **K**, particularly predominant in

CAD-deficient angiosperms (76a, 113). Incorporation profiles of hydroxycinnamyl aldehydes **M2** provide further evidence that lignification reactions are under simple chemical control. In tobacco, sinapyl aldehyde **M2S** is found 8–O–4-coupled (in structures **K**) to both **G** and **S** units, whereas coniferyl aldehyde **M2G** is only found cross-coupled to **S** units. Coniferyl aldehyde has not been successfully coupled to guaiacyl models in vitro, although the 8–O–4-coupled dehydrodimer can be obtained. An important corollary is that hydroxycinnamyl aldehydes are incorporated intimately into angiosperm **GS** lignins but only poorly into gymnosperm **G** lignin.

The most striking example of lignins incorporating substantial quantities of a monomer derived from truncated monolignol biosynthesis is in caffeic acid-*O*-methyltransferase (COMT)-deficient angiosperms (113). Plants severely depleted in COMT produce little sinapyl alcohol **M1S** but essentially substitute it with a monomer derived from its unmethylated precursor, 5-hydroxyconiferyl alcohol **M15H**. The incorporation is typically end-wise, but the *o*-diphenol results in novel cyclic structures, benzodioxanes **J**, in the lignin (112). As with other products, such units can be found at very low levels in normal plants.

VARIABILITY AND TOPOCHEMISTRY

Lignin deposition is one of the final stages of xylem cell differentiation and mainly takes place during secondary thickening of the cell wall (37). Generally, secondary cell walls consist of three layers: the outer (S1), middle (S2), and inner (S3). Lignin deposition proceeds in different phases, each preceded by the deposition of carbohydrates, and starts at the cell corners in the region of the middle lamella and the primary wall when S1 formation has initiated. When the formation of the polysaccharide matrix of the S2 layer is completed, lignification proceeds through the secondary wall. The bulk of lignin is deposited after cellulose and hemicellulose have been deposited in the S3 layer. Generally, lignin concentration is higher in the middle lamella and cell corners than in the S2 secondary wall (11, 37, 124). However, because it occupies a larger portion of the wall, the secondary wall has the highest lignin content. Secondary walls of vessels generally have a higher lignin content than those of fibers. Environmental conditions also influence lignin amount and composition; secondary cell walls of angiosperm tension wood are characterized by the presence of an unlignified gelatinous layer, which is composed of highly crystalline cellulose, whereas lignin distribution is normal in the rest of the secondary cell wall. By contrast, the S2 layer of gymnosperm compression wood is characterized by a highly lignified ring (the S2L layer) (37).

Microautoradiography and UV-microspectrometry have shown that the three monolignols are incorporated at different stages of cell wall formation. Typically, **H** units are deposited first, followed by **G** units, and **S** units still later in angiosperms (37, 139). Lignin in vessels is generally enriched in **G** units, whereas lignin in fibers is typically enriched in **S** units (124). A large proportion of **S** units is also found in secondary walls of ray parenchyma (44). In gymnosperms, the lignin deposited in compression wood is enriched in **H** units (141). In

gramineous monocotyledons, lignin incorporates significant amounts of hydroxycinnamate esters. Ferulate-polysaccharide esters **M4G** (and minor amounts of the *p*-coumarate analogs **M4H**) are rapidly deposited at the early stages of lignification. *p*-Coumarates **Y3**, acylating lignin side-chains mainly on **S** units (85), are deposited throughout lignification (61), implicating the involvement of acylated monomers **M11**. The difference in timing of monolignol deposition is associated with variations in lignin condensation in the individual cell wall layers, as shown by immunocytochemistry with antibodies raised against pure **H**, pure **G**, or mixed **GS** synthetic lignins (23, 72).

The chemical nature of the carbohydrate matrix and the orientation of the cellulose microfibrils influence lignin deposition. In the middle lamella and the primary wall, lignin forms spherical structures, whereas in the secondary wall, lignin forms lamellae that follow the orientation of the microfibrils (5, 37, 123). During deposition, lignin may form chemical bonds with the hemicellulose component in the wall and gradually eliminates water, forming a hydrophobic environment.

From these data, one can conclude that lignin deposition, and the relative incorporation of the different monolignols into the polymer, are spatially and temporally regulated. The mechanisms controlling this process are not yet fully resolved but are likely governed by the interplay between the spatio-temporal expression of monolignol biosynthetic genes, the kinetics of monolignol delivery to the cell wall, and the chemistry of monolignol coupling to the growing polymer in the complex macromolecular environment of the cell wall.

MONOLIGNOL BIOSYNTHESIS

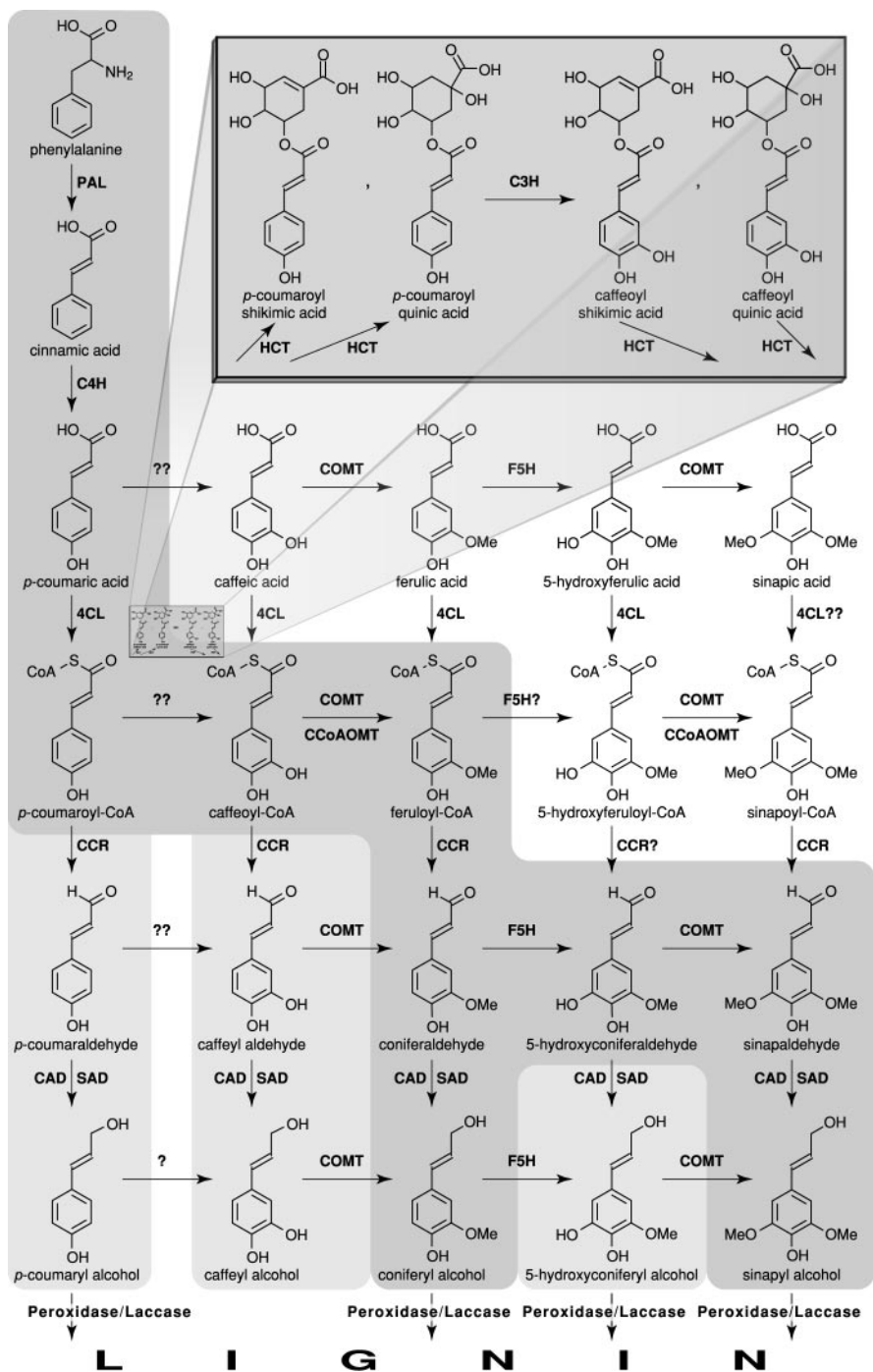
The biosynthesis of the monolignols starts with the deamination of phenylalanine and involves successive hydroxylation reactions of the aromatic ring, followed by phenolic *O*-methylation and conversion of the side-chain carboxyl to an alcoholic group. Monolignol biosynthesis can be most easily understood starting with the metabolic grid presented in Figure 3, which includes all possible enzymatic conversions that have been shown by *in vitro* experiments. Researchers have long thought that the hydroxylation and methylation reactions occur at the level of the cinnamic acids and that *p*-coumaric, ferulic, and sinapic acid are subsequently converted to the corresponding monolignols by the sequential action of 4-coumarate:CoA ligase (4CL), CCR, and CAD. However, a number of *in vitro* enzymatic assays with heterologously produced enzymes, the identification of novel genes implicated in the pathway, and analyses of mutant and transgenic plants modified in monolignol biosynthesis have cast doubt on this route, and the pathway had to be redrawn (67). For clarity, we present only the key arguments that have led to the currently most favored pathway.

The first revision of the pathway arose from the observation that expression of caffeoyl-CoA *O*-methyltransferase (CCoAOMT) coincided with lignin deposition in differentiating tracheary elements in zinnia (148). CCoAOMT methylates

caffeoyl-CoA and 5-hydroxyferuloyl-CoA to produce feruloyl-CoA and sinapoyl-CoA in vitro. Further research suggested the existence of a route from *p*-coumaroyl-CoA to caffeoyl-CoA (150). Subsequently, feeding experiments with radiolabeled monolignol glucosides showed that hydroxylation and methylation of the aromatic C₃ and C₅ positions could also occur at the aldehyde or alcohol level (28, 89, 90), indicating the existence of enzymes able to catalyze these conversions. Re-evaluating the substrate preference of ferulate 5-hydroxylase (F5H) showed that the preferential substrate was not ferulic acid, but coniferaldehyde and coniferyl alcohol (68, 98). Similarly, the products of F5H-catalyzed hydroxylation, 5-hydroxyconiferaldehyde, and 5-hydroxyconiferyl alcohol proved to be good substrates for COMT, whereas caffeic acid was a poor substrate (68, 83, 98, 100). Together, these data presented evidence that the aromatic C₅ position is hydroxylated and methylated preferentially at the cinnamaldehyde or cinnamyl alcohol level and that the predominant role for CCR is the reduction of feruloyl-CoA to coniferaldehyde. In a variety of species, caffeoyl aldehyde and caffeoyl alcohol are also efficiently *O*-methylated, in agreement with the radiotracer studies (27a, 54, 55, 89, 100, 151). Caffeoyl aldehyde is probably synthesized from caffeoyl-CoA by CCR (54). Hence, coniferaldehyde may be synthesized from feruloyl-CoA or from caffeoyl aldehyde, depending on differences in substrate specificities between enzyme isoforms and depending on the species (151).

CAD is a multifunctional enzyme that catalyzes the final reduction of the cinnamaldehydes to the corresponding alcohols, at least in vitro. However, a CAD homolog from aspen, sinapyl alcohol dehydrogenase (SAD), that preferentially reduces sinapaldehyde to sinapyl alcohol was identified. This homolog is co-expressed with F5H and COMT and co-localizes with S lignin in aspen (82). Aspen CAD preferentially reduces coniferaldehyde; therefore, SAD may be the enzyme responsible for the final step in the biosynthesis of sinapyl alcohol (82).

The enzyme that was long thought to convert *p*-coumaric acid into caffeic acid has only recently been cloned from *Arabidopsis* and shown to be a cytochrome P450-dependent monooxygenase (47, 127). It is interesting to note that enzymatic assays have demonstrated that the shikimate and quinate esters of *p*-coumaric acid are the preferred substrates for *p*-coumarate 3-hydroxylase (C3H) and that neither *p*-coumaric acid, *p*-coumaroyl-CoA, *p*-coumaraldehyde, *p*-coumaryl alcohol, nor the 1-*O*-glucose ester and the 4-*O*-glucoside of *p*-coumaric acid are good substrates (47, 95a, 127). By incorporating C3H into the scheme for monolignol biosynthesis, at least in *Arabidopsis*, *p*-coumarate is first converted to *p*-coumaroyl-CoA by 4CL, with subsequent conversion to *p*-coumaroyl-shikimate and *p*-coumaroyl-quininate, the substrates for C3H, by *p*-hydroxycinnamoyl-CoA:D-quininate- (CQT) or *p*-hydroxycinnamoyl-CoA:shikimate *p*-hydroxycinnamoyltransferase (CST) (127). These enzymes, described as reversible enzymes, can convert caffeoyl-shikimate or caffeoyl-quininate (chlorogenic acid) into caffeoyl-CoA, the substrate for CCoAOMT (127, 144). Recently, a reversible acyltransferase with both CQT and CST activity, designated HCT, has been purified and the corresponding gene cloned from tobacco (62b). Taken together, these data argue that none of



the C₃ and C₅ substitutions of the aromatic ring take place at the cinnamic acid level in monolignol biosynthesis.

For most of the enzymes described above, multiple isoforms that are differentially expressed during development and upon environmental cues (27, 66, 79, 84a) exist that may have different kinetics and substrate preferences (39, 59, 151). Certain paths in the grid are therefore expected to be kinetically favored in given cell types or environmental conditions, allowing for metabolic flexibility (Figure 3). In this respect, a continuing controversy concerns the role of sinapic acid in monolignol biosynthesis. Whereas 4CL isoforms of most plant species analyzed use *p*-coumarate, caffeate, and ferulate as substrates, but not sinapate, isoforms of some plants are able to convert sinapate into sinapoyl-CoA (84a), leaving the possibility that in particular plants monolignols may be synthesized via the acid pathway (147a).

Another level of complexity is that pathway intermediates may affect the synthesis or activity of certain enzymes in the pathway. Cinnamic acid inhibits phenylalanine ammonia-lyase (*PAL*) expression at the transcriptional (13, 91) and posttranslational level (16) and induces the activity of CQT (77). Accordingly, downregulation of cinnamate 4-hydroxylase (*C4H*) in transgenic tobacco reduces *PAL* activity by feedback modulation (13). Phenylalanine concentrations also have a profound effect on flux through the pathway. Feeding lignifying *Pinus taeda* cell suspension cultures with phenylalanine increases *p*-coumaryl and coniferyl alcohol synthesis and *PAL*, *4CL*, *CCoAOMT*, and *CCR* transcript levels, but only slightly upregulates those of *C4H* and *C3H* (4).

In vitro enzymatic experiments have shown that 5-hydroxyconiferaldehyde is a competitive inhibitor of caffeic and 5-hydroxyferulic acid methylation and that coniferaldehyde is a noncompetitive inhibitor of ferulate 5-hydroxylation, corroborating the conclusion that C₃ and C₅ substitutions in monolignol biosynthesis do not take place at the cinnamic acid level (83, 98). In aspen, coniferaldehyde

←
Figure 3 Phenylpropanoid and monolignol biosynthetic pathways. Based on lignin compositional analyses, enzymatic assays, and transgenic plants, the black route toward the production of monolignols is expected to be most favored in angiosperms. The gray routes also occur, depending on the species and conditions. The route from caffeic acid to sinapic acid and down to 5-hydroxyferuloyl-CoA and sinapoyl-CoA (*white part*) does not play a significant role in monolignol biosynthesis. CAD, cinnamyl alcohol dehydrogenase; 4CL, 4-coumarate:CoA ligase; C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid *O*-methyltransferase; HCT, *p*-hydroxycinnamoyl-CoA: quinate shikimate *p*-hydroxycinnamoyltransferase; F5H, ferulate 5-hydroxylase; PAL, phenylalanine ammonia-lyase; SAD, sinapyl alcohol dehydrogenase. ?, conversion demonstrated; ??, direct conversion not convincingly demonstrated; 4CL??, some species have 4CL activity toward sinapic acid; CCR? and F5H?, substrate not tested; others, enzymatic activity shown in vitro.

is additionally a competitive inhibitor of the sinapaldehyde reduction by CAD, whereas sinapaldehyde strongly inhibits the coniferaldehyde reduction by SAD, indicating that SAD is predominantly involved in sinapyl and CAD in coniferyl alcohol biosynthesis (82). Caffeic acid competitively inhibits the 4CL activation of *p*-coumaric acid (59), and feedback regulation by hydroxycinnamic acid levels has also been suggested for CCoAOMT (70).

Furthermore, transgenic plants have shown that COMT downregulation in tobacco induces a strong increase in CCR activity and has a negative effect on the production of CCoAOMT (104), indicating additional regulatory mechanisms controlling the flux through the pathway.

TRANSGENIC PLANTS

In vitro assays of individual pathway enzymes and radiolabeling experiments have been instructive in dissecting the monolignol biosynthetic pathway, but they are insufficient to comprehend this pathway's complexity in vivo. Transgenic plants provide a picture of the gross alterations on lignin amount, composition, and primary structure and on the phenotypic effects caused by altering the expression of a single gene. Hence, one can obtain novel insight into regulatory aspects of the pathway and on the structure and biological roles of lignin.

Transgenic plants or mutants with modified expression of all monolignol biosynthesis genes described above, except HCT, have been studied in detail (36) (for an extensive table, see Supplemental Table: Follow the Supplemental Material link from the Annual Reviews home page at <http://www.annualreviews.org/>). Below, we focus on the main results that have contributed to our understanding of lignin biosynthesis and structure.

Downregulation of PAL and C4H in tobacco largely reduces lignin content, consistent with their key positions at the entry point to phenylpropanoid biosynthesis (8, 43, 130). Surprisingly, lignin composition is oppositely affected in these transgenic tobacco lines. PAL downregulation reduces mainly **G** units, whereas C4H downregulation reduces mainly **S** units in lignin, an observation that cannot easily be explained by the pathway presented in Figure 3. Three possible explanations have been proposed for this apparent contradiction (36): (a) The pathway to **G** lignin may bypass C4H, (b) C4H still catalyzes reactions other than the 4-hydroxylation of cinnamic acid, or (c) C4H may be part of a metabolic channel committed to **S** lignin biosynthesis. C3H, C4H, and F5H are all membrane-bound P450 enzymes, with an endoplasmic reticulum anchor domain and, as such, poised well to assemble multienzyme complexes at the outer face of the endoplasmic reticulum membrane (25, 119, 127). Until now, however, metabolic channeling has only been demonstrated from phenylalanine to *p*-coumarate, involving a microsomal PAL isoform (118).

Overexpression of PAL in tobacco leads to increased levels of chlorogenic acid in leaves (8, 64), whereas downregulation of PAL and C4H decreases chlorogenic acid content (8, 13), providing a link between the role of C3H in 3-hydroxylation at the level of shikimate or quinate esters and lignin (46, 47, 127).

Downregulation of 4CL leads to a reduction in lignin content in tobacco, *Arabidopsis*, and aspen (65, 74, 80) and to a higher amount of cell wall-bound hydroxycinnamic acids (*p*-coumaric, ferulic, and sinapic acid) in tobacco and poplar (65, 74). The effects on **S/G** lignin composition are contradictory. In tobacco, a reduction in **S** units is reported (74, 75); in *Arabidopsis*, only **G** units are reduced (80); and in transgenic aspen, the **S/G** ratio is similar to that of the control (65). This differential effect on lignin composition may be explained by the existence of multiple isoforms of 4CL with distinct substrate specificities and various functions that are differentially affected in the transgenic plants (3, 39, 59, 65, 80).

The *Arabidopsis reduced epidermal fluorescence* (*ref8*) mutant, defective in *C3H*, has a strongly reduced lignin content and accumulates a range of soluble *p*-coumarate esters instead of sinapoyl malate. The lignin of this mutant is almost entirely composed of **H** units and contains large amounts of esterified *p*-coumaric acid. These data indicate that *C3H* is a major control point in the production of **C**₃- and **C**₅-substituted phenylpropanoids and that it is likely the sole 3-hydroxylase in the phenylpropanoid pathway (46).

Downregulation of CCoAOMT results in less lignin, and in an increased **S/G** ratio mainly because of a reduction in **G** units (55, 93, 105, 149). In alfalfa, downregulation of CCoAOMT reduces **G** lignin but does not affect **S** lignin biosynthesis, suggesting that, in alfalfa, CCoAOMT is not essential in **S** unit biosynthesis *in vivo* and COMT is involved in the 3-methylation step, in agreement with the substrate specificity of alfalfa COMT allowing the methylation of caffeoyl aldehyde and caffeoyl alcohol (27a, 55, 100). Metabolic profiling of stem tissues revealed the accumulation of soluble *O*- β -D-glucosides of vanillic, caffeic, and sinapic acids in transgenic poplar (93), and caffeoyl- β -D-glucoside in alfalfa (55); all are compounds that probably derive from caffeic acid upon de-esterification of caffeoyl-CoA by a thioesterase. In poplar, elevated levels of free and wall-bound *p*-hydroxybenzoic acid **Y2** were found (93, 149), and lignin is less condensed and less cross-linked (149).

Downregulation of COMT to low activity levels reduces lignin content in alfalfa, maize, and poplar by 30%, 30%, and 17%, respectively. Consistent with the predominant role for COMT in methylating the **C**₅ hydroxyl group, the most striking effect of COMT deficiency is the reduction in **S** units and the incorporation of 5-hydroxyconiferyl alcohol **M15H** into the lignin polymer (6, 24, 55, 73, 112, 113, 145, 150). Accordingly, the lignin of transgenic poplar and alfalfa downregulated for COMT is characterized by a reduced frequency of bonds involving **S** units (55, 73, 78). COMT-downregulated poplars have more 5-5 **D** and less β - β **C** structures in lignin (78), and they have high amounts of novel benzodioxane structures **J** derived from incorporation of the 5-hydroxyconiferyl alcohol **M15H** (112) (see Supplemental Three-Dimensional View of the altered lignin structure in a COMT-deficient poplar).

In tobacco, downregulation of CCR has a marked effect on both lignin content and lignin composition (106, 110). The **S/G** ratio is increased mainly because of a reduction in **G** units, but lignin is more condensed. A higher amount of tyramine ferulate **M8G** is incorporated as an integral component of the polymer.

In addition, increased amounts of cell wall-bound phenolics, such as ferulic acid, sinapic acid, acetosyringone, and tyramine ferulate **M8G**, were released by mild alkaline hydrolysis of the cell wall. Tyramine ferulate **M8G** is likely the sink for the anticipated buildup of feruloyl-CoA. The higher levels of hydroxycinnamates may have contributed to the abnormally high degree of cross-linking.

Lignin of the *Arabidopsis* mutant defective in *F5H* (*fah-1*) is characterized by the absence of **S** units and enhanced phenylcoumaran **B** and dibenzodioxocin structures **D2** (26, 88), whereas upregulation of *F5H* in *Arabidopsis*, tobacco, and poplar results in lignin almost entirely constituted of **S** units, therefore containing no phenylcoumaran **B** or dibenzodioxocin **D2** structures (48, 88, 92). It is interesting to note that benzodioxane **J** structures, which are found in plants with suppressed *COMT* expression, are also present in the lignin of *F5H*-overexpressing *Arabidopsis* plants, probably because of the increased flux to 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol **M15H** without an associated increase in *COMT* activity (113). These data show that *F5H* plays a major role in 5-hydroxylation and that it is possible to modulate the **S/G** ratio in plants from one extreme to the other.

Although *CAD* catalyzes the last step in monolignol biosynthesis, lignin content is only slightly affected in most *CAD*-deficient plants (9, 10, 58, 62, 78, 87, 103, 134). This can, at least in part, be explained by the incorporation of other phenolics that compensate for the reduced availability of monolignols for polymerization. Indeed, downregulation of *CAD* in tobacco and poplar results in coniferaldehyde and sinapaldehyde incorporation into the lignin polymer (76a, 113). In pine, a *CAD* mutation has been associated with an increased amount of coniferaldehyde **X2G** and unanticipated dihydroconiferyl alcohol **X5G** units (114, 128). The *CAD*-deficient *bm1* mutant of maize has a 20% reduced lignin content (57) and stains more strongly for aldehydes; however, no aldehyde resonances were seen in nuclear magnetic resonance spectra (107).

The expression of two or three genes of the monolignol biosynthetic pathway has been altered either by crossing single transformants downregulated for particular genes [*COMT* and *CCR* (104), *COMT* and *CCoAOMT* (105), *CAD* and *CCR* (22, 23)], by double transformation [*COMT* and *CCoAOMT* (55, 150)], or by introduction of a chimeric construct consisting of fragments of three genes [*COMT*, *CCR*, and *CAD* (1)] (for additional information, see Supplemental Table).

A striking characteristic of transgenic plants downregulated for 4CL, *CCoAOMT*, *CCR*, *CAD*, and *COMT* is the reddish or brownish discoloration of the xylem tissues, initially observed in the maize “brown-midrib” (*bm*) mutants (see Supplemental Table) (Figure 4). The reddish coloration of *CAD*- and *COMT*-deficient plants has been attributed to the incorporation of cinnamaldehydes in the polymer; synthetic DHPs of coniferyl alcohol and coniferaldehyde also form a red polymer (62a). However, in plants downregulated for 4CL, *CCoAOMT*, and *CCR*, the incorporation of other phenolics into the lignin polymer is likely the cause of the xylem discoloration.

Taken together, the results obtained by analyzing transgenic plants modified in monolignol biosynthesis have demonstrated that plants can tolerate large variations

in lignin content and composition, that monomers other than *p*-coumaryl, coniferyl, and sinapyl alcohol are incorporated into the lignin polymer, and that the copolymerization of these uncommon monomers may result in novel lignin structures. These data show that the lignin polymer is extremely flexible in its composition.

Besides having contributed to our understanding of lignin biosynthesis and structure, the same transgenic lines have also demonstrated that lignin is important for the structural integrity of the cell wall. Plants downregulated in C3H, CCoAOMT, and CCR all had reductions in lignin content associated with a collapse of the vessels and altered growth, phenotypes that may significantly vary according to developmental and environmental conditions (104, 105). Such collapsed vessels have been studied in detail in CCR-downregulated tobacco and *Arabidopsis irx4* mutants and have an expanded S2 secondary wall with individualized cellulose microfibrils (23, 71, 104), indicating an important role for lignin as cohesive between cellulose microfibrils. However, in some cases, reduced lignin content is not associated with growth abnormalities (80) and, in the case of 4CL downregulation in aspen, even with increased growth, showing that lignin content per se is not essential for structural integrity of the cell wall and that the reduced lignin content can be compensated by other cell wall constituents (65). The increase in cellulose coupled to a decrease in lignin observed in 4CL-downregulated poplars does not seem to be a general phenomenon; *irx4* mutants defective in CCR have less lignin but no increase in cellulose (71).

TRANSPORT OF MONOLIGNOLS

After their synthesis, the lignin precursors are transported to the cell wall where they are oxidized and polymerized. In gymnosperms and some angiosperms, monolignol 4-*O*- β -D-glucosides accumulate to high levels in the cambial tissues (132). It has been hypothesized that these monolignol glucosides are storage or transport forms of the monolignols and that a uridine diphosphate glucose (UDPG) coniferyl alcohol glucosyl transferase (132), together with coniferin- β -glucosidase (CG), may regulate storage and mobilization of monolignols for lignan and/or lignin biosynthesis (34, 125). Whether these glucosides are transported via Golgi-derived vesicles or through direct plasma membrane pumping by ABC transporters is still unknown (125).

By taking advantage of the completed sequence of the *Arabidopsis* genome and by combining bioinformatics with a biochemical approach, two UDPG-glycosyl transferases (UGTs) that were able to 4-*O*-glucosylate sinapyl alcohol into syringin were identified, one of which could also glucosylate coniferyl alcohol to coniferin in vitro (84). Identifying these UGT genes enables one to study the role of monolignol glucosides in vivo by reverse-genetics.

If monolignol glucosides were indeed the monolignol transport (or storage) forms, the aglycon would have to be liberated by a CG prior to dehydrogenation. Based on peptide sequence information obtained from a purified lodgepole pine CG, the corresponding cDNA has been cloned and shown to encode a secretory

family-1 glycosyl hydrolase (34, 35). CG has been immunolocalized to xylem secondary walls (125). However, no genetic evidence for its role in lignification has been obtained yet.

According to early studies with radiolabeled monolignol precursors, lignification of the cell wall has been hypothesized to proceed after cell death (102). These findings are now supported by experiments with the zinnia cell system showing that lignification of tracheary elements that have undergone programmed cell death still progresses by supply of monolignols from the surrounding xylem parenchyma cells (63).

DEHYDROGENATION

After transport of the monolignols to the cell wall, lignin is formed through dehydrogenative polymerization of the monolignols (29). The dehydrogenation to monolignol radicals has been attributed to different classes of proteins, such as peroxidases, laccases, polyphenol oxidases, and coniferyl alcohol oxidase. Which of these enzymes or a combination thereof are responsible for the dehydrogenation of the monolignols in planta and whether monolignol oxidation occurs through redox shuttle-mediated oxidation are still unclear (97).

Peroxidases use hydrogen peroxide (H_2O_2) to oxidize their substrates (29). How H_2O_2 is generated in the cell wall is still a matter of debate. Evidence is emerging for a role for an NADPH oxidase in lignifying tissues, which would supply H_2O_2 for monolignol oxidation (96, 121). However, a copper amine oxidase (CuAO) that generates H_2O_2 by oxidizing putrescine has also been co-localized with lignin staining and peroxidase activity in tracheary elements of *Arabidopsis* (94). Oxalate oxidase is another enzyme possibly involved in H_2O_2 generation (17), and peroxidase itself is able to generate H_2O_2 from a variety of reducing substrates, such as cysteine, glutathione, NADPH, ascorbate, and indole-3-acetic acid (15, 29, 45).

The high number of peroxidase genes [73 in the *Arabidopsis* genome (142)] is one reason why isozymes that are responsible for monolignol oxidation in vivo cannot be identified easily. In addition, most peroxidases isolated from a variety of species catalyze very similar reactions, such as the oxidation of the monolignol coniferyl alcohol. Thus, oxidation of coniferyl alcohol is no proof for peroxidase involvement in lignin polymerization. Although several anionic and cationic peroxidases are expressed in lignifying cells, and some can generate lignin ectopically in planta upon overexpression (30, 40–42, 99, 108, 120), peroxidase involvement in the developmentally regulated deposition of lignin still needs to be unambiguously demonstrated.

Laccases (*p*-diphenol: O_2 oxidoreductases) are copper-containing, cell wall-localized glycoproteins that are encoded by multigene families in plants. In contrast to peroxidases, laccases consume O_2 instead of H_2O_2 to oxidize the monolignols. Laccases of a variety of species are expressed in lignifying cells (7, 38, 117, 133).

However, downregulation of laccase in aspen and yellow poplar did not affect lignin content or composition (33, 116).

POLYMERIZATION

Radical Generation and Radical Coupling

After their dehydrogenation, the radicals, which are relatively stable owing to electron delocalization that provides single-electron density to the side-chain β position, are coupled. The most important reaction is cross-coupling to the growing polymer to extend the complex three-dimensional lignin network (Figure 2). But, such coupling reactions are radical quenching. Each extension of the polymer requires new radicals on each of the two coupling partners. Radicals on the growing lignin polymer are thought to be generated by radical transfer from monolignols or other intermediaries. *p*-Coumarates function as efficient intermediaries in sinapyl alcohol-coupling reactions; they are rapidly oxidized by peroxidases, and then transfer the radical to sinapyl alcohol, which forms a more stable radical (59a, 136). As a result, sinapyl alcohol is far more rapidly oxidized in the presence of *p*-coumarates; the *p*-coumarates are not coupled until all of the sinapyl alcohol is depleted. This radical transfer step is assumably one of the functions of monolignol *p*-coumarate esters **M11** in maize, where the peroxidases seem to be poor at oxidizing sinapyl alcohol directly. Similar radical transfer mechanisms can be envisioned between the monolignols and the growing polymer, i.e., the monolignols may act as the radical shuttles. When a monolignol radical encounters a polymer radical, it may cross-couple with it, but when the polymer is not electron-deficient, radical transfer may occur and the monolignol will diffuse back to the peroxidase/laccase to be reoxidized. Alternatively, redox shuttles, such as an $\text{Mn}^{2+}/\text{Mn}^{3+}$ system (97), may be involved.

Polymerization Process

The actual process of polymer formation, lignification, occurs without the rigid biochemical controls seen in the biosynthesis of the precursor monolignols, giving rise to a unique class of polymers. Lignins are racemic (115), deriving from radical coupling reactions under chemical (but no apparent biochemical control) between phenolic radicals in an essentially combinatorial fashion.

Much of what is known of the radical-coupling process and the parameters that determine the frequency of interunit bonds and the structure of lignin has been obtained via synthetic dehydrogenation polymers (DHPs). Lignin-like polymers can be artificially synthesized in vitro by dehydropolymerization of lignin precursors, using peroxidase/ H_2O_2 or laccase/ O_2 as oxidizing agents (126). The Zutropf DHPs, formed by adding lignin precursors slowly and continuously to a solution containing H_2O_2 and peroxidase, are called end-wise polymers and structurally resemble

isolated wood lignins more closely than Zulauf DHPs or bulk polymers, which are formed by adding the precursors in a single batch (49). Such DHP experiments have indicated that lignin structure depends on the supply rate of the monomers, the rate of radical generation (2, 135, 138), the presence of polysaccharides in the DHP mix (137, 138, 140), and the presence of the growing lignin polymer (53).

The accepted model for lignin polymerization, based on simple chemically controlled combinatorial coupling reactions, was recently challenged. According to Lewis' group (31), the macromolecular assembly of lignin is not based on "random coupling" of monolignols. Instead, this group proposes a strong biological control over the outcome of phenoxy radical coupling *in vivo*. The new theory arose from the discovery of a fascinating class of dirigent proteins implicated in lignan biosynthesis (32); lignans are dehydrodimers of monolignols and are typically optically active. The first such dirigent protein discovered guided the dimerization of coniferyl alcohol radicals to produce an optically active lignan, pinoresinol. The corresponding gene was cloned and shown to encode a cell wall-localized protein. The finding was extrapolated to lignification, suggesting that such proteins would logically be responsible for specifying the exact structure of the lignin polymer, bringing lignins in line with proteins and polysaccharides that are more carefully biosynthesized (31, 81). However, no strong arguments invoke a role for dirigents in lignification, and numerous facts do not fit the proposed model for absolute structural control over lignification (60, 113, 122, 128). Reverse genetics will tell whether dirigents play any role in lignin biosynthesis in addition to their involvement in lignan biosynthesis.

Nucleation Sites

As discussed above, lignin is first deposited in the middle lamella and the cell corners of the primary wall after the formation of the secondary wall has started, at the so-called nucleation sites, from which the lignin polymers can grow. The nature of these nucleation sites is unknown. Ferulates, conjugated to polysaccharides, and their dehydrodimers are well established as being incorporated into grass lignins. There is some evidence that ferulates and diferulates may act as attachment sites for monolignols (109). Structural cell wall proteins rich in aromatic residues, such as glycine-rich proteins (76), may have a similar function. It is interesting to note that several apoplastic peroxidases from zucchini and horseradish bind pectin in their Ca^{2+} -induced conformation (101); one such peroxidase has been cloned (19, 20). Given that the middle lamella and the cell corners are rich in Ca^{2+} pectate (21) and are the first sites to be lignified, Ca^{2+} pectate-bound peroxidases may conceivably play a role in the spatial control of lignin deposition, and changes in Ca^{2+} and H^+ concentrations may modulate the location of these peroxidases (19).

The negatively charged pectins are also good binding sites for polyamines (19) and, hence, may be suitable sites for H_2O_2 generation by polyamine oxidases (94). It is tempting to speculate that pectin-binding peroxidases and polyamine oxidases may act locally in the early stages of lignin deposition both for H_2O_2 generation and

oxidation of monolignols, cinnamic acids bound to polysaccharides or polyamines, or aromatic residues on certain proteins, such as glycine-rich proteins (76).

BIOTECHNOLOGY

Lignin biosynthesis is an active field of research partly because of its economic relevance. For the production of high-quality paper, lignin needs to be extracted from the pulp by expensive and environmentally hazardous processes requiring large amounts of energy and chemicals. For the paper industry, it would be beneficial to process wood with either less lignin or lignin with an altered chemical reactivity.

The first attempts to reduce lignin content involved downregulation of CAD and COMT. Surprisingly, large reductions in CAD activity only slightly reduced lignin content because the plants were able to circumvent the block in CAD activity by shipping its substrates, the cinnamaldehydes, to the cell wall for polymerization. An additional surprise was that the altered lignin structure did not affect overall plant growth and development. These data show how adaptable plants are in building their cell wall (10, 58, 62, 76a, 78, 87, 134). Interestingly, chemical pulping experiments with wood harvested from 4-year-old, field-grown transgenic poplars downregulated for CAD have demonstrated that the modifications in lignin structure result in an altered chemical reactivity, which reduces the consumption of chemicals needed to remove lignin from the pulp. The pulp yield was simultaneously enhanced. These data also show that significant improvements in pulping efficiency can be achieved without strong reductions in lignin content (103). Similar results were obtained in chemical pulping experiments with wood from transgenic tobacco plants downregulated for CAD and CCR (95b).

Another unanticipated observation was that downregulation of COMT primarily affected the biosynthesis of **S** units, a first indication that the pathway that had been described in textbooks for many years was wrong (6, 145). In these plants, a novel unit **M15H** was copolymerized in the polymer and resulted in new types of chemical bonds **J**, again demonstrating the extraordinary flexibility of the lignin polymer. Pulping experiments of wood from field-grown COMT-downregulated poplars show that lignin of these plants is more difficult to extract from the pulp, presumably because of the reduced synthesis of **S** units and the presence of etherified units derived from 5-hydroxyconiferyl alcohol **M15H**, which are not cleavable in base (73, 78, 103). Although these plants are not very interesting for the chemical pulp industry, the results demonstrated that lignin composition plays an important role in lignin extractability and that it can be modified without affecting plant viability.

An additionally appealing objective is to engineer **S** lignin in gymnosperms. The rationale behind this objective is that a lignin polymer enriched in **S** units is less cross-linked than lignin rich in **G** units. As discussed above, results from enzymatic assays and transgenic plants have demonstrated that angiosperm F5H, COMT, and SAD are the enzymes responsible for **S** biosynthesis; upregulation of F5H results in lignin almost entirely composed of **S** units (48, 88, 92), whereas F5H deficiency

essentially abolishes **S** unit biosynthesis (26, 88). Likewise, downregulation of COMT limits **S** unit synthesis. No transgenic plants have been made yet for SAD, but enzymatic assays indicate it preferentially reduces sinapaldehyde, the last step in the biosynthesis of sinapyl alcohol (82). Through the combined expression of these three angiosperm genes (*F5H*, *COMT*, and *SAD*) in gymnosperms, it should be possible to divert coniferaldehyde and coniferyl alcohol toward the synthesis of **S** units and to improve lignin extractability.

Transgenic poplars downregulated for 4CL also hold great promise for the pulp industry; they have less lignin and more cellulose, and they grow taller for still unknown reasons. These three factors may significantly affect pulp yield (65).

Lignin also inhibits forage digestibility by ruminants (147). Downregulation of COMT, PAL, and CCoAOMT resulted in improved digestibility (56). Tobacco and alfalfa, downregulated for CAD, had higher in situ disappearance of cell walls (9, 12), and cell walls prepared from the *Arabidopsis ref8* mutant deficient in C3H and having lignin almost entirely composed of **H** units are more susceptible to polysaccharide hydrolases than wild type (46). Experiments have shown that **H**, **G**, and **S** lignins have similar effects on wall degradability when other cell wall factors are kept constant (52). Therefore, altering **S/G** composition per se probably does not modify digestibility, but lignin content (131), other compositional and structural factors (such as cross-linking), or overall changes in agronomic characteristics (such as leaf-to-stem ratio).

Lignin research may well be validated in breeding programs. Several quantitative trait loci for lignin composition in eucalyptus coincide with map positions of genes involved in lignin biosynthesis (51), and maize *bm* mutants with altered agronomic performance have defects in monolignol biosynthesis genes (57, 146), paving the way for the use of molecular markers in marker-assisted selection programs.

CONCLUSIONS AND PERSPECTIVES

The analysis of transgenic plants and mutants has significantly contributed to understanding the *in vivo* role of the enzymes of the lignin biosynthetic pathway and, in several cases, has demonstrated that *in vitro* enzymatic assays with a set of presumed substrates can be misleading. Nevertheless, several factors call for a careful interpretation of the results obtained with transgenic plants and mutants too. Altering the activity of a single enzyme of the pathway may modify the level of pathway intermediates, thus affecting the expression of other genes at the transcriptional or posttranslational level (4, 13, 14, 59, 82, 104). Additionally, the expression of unknown functional homologs of the gene under study may be affected. Hence, the pleiotropic phenotype displayed by several of the mutant lines under study is the result of both direct and indirect consequences of the mutation; the latter is the product of a number of unknown molecular events.

With the advent of microarray technology, the full spectrum of transcriptional events caused by altering the expression of a single gene will be revealed. Coupled

with metabolite profiling of the same tissues through high-performance liquid chromatography and gas chromatography combined with mass spectrometry (143), and reverse-genetics approaches, these data should deepen our understanding of the interrelationships between biosynthetic pathways and plant development.

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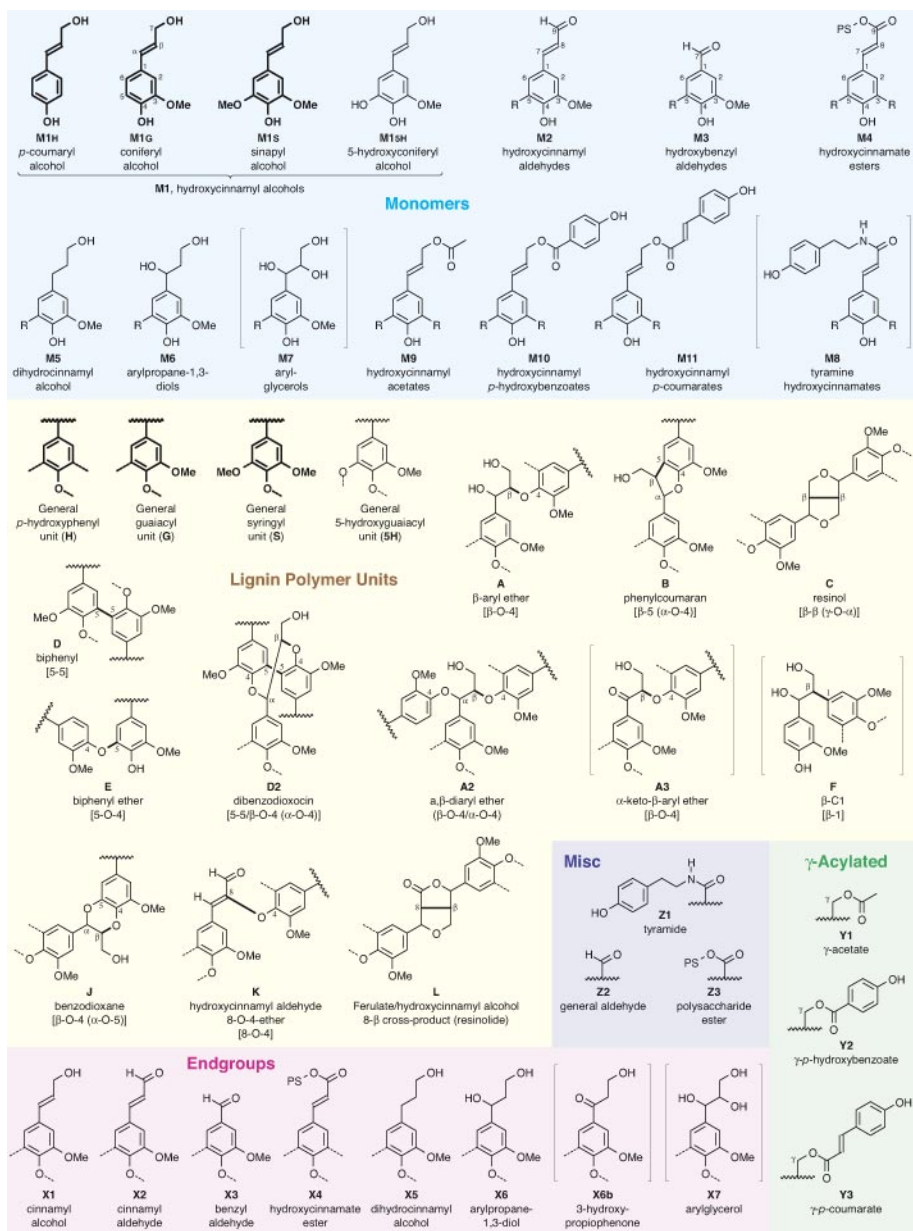
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Figure 1 Lignin monomers and structures in the polymer. (*Monomers*) Lignins derive primarily from the three traditional monolignols, the hydroxycinnamyl alcohols: **M1H**, **M1G**, and **M1S**. The fourth hydroxycinnamyl alcohol, **M15H**, is a significant monomer in COMT-deficient plants. Aldehydes **M2** and **M3** probably incorporate into all lignins and increasingly so in CAD-deficient plants. Hydroxycinnamate esters **M4**, particularly ferulates **M4G**, incorporate into lignins in grasses. Dihydroconiferyl alcohol **M5G**, and the guaiacylpropane-1,3-diol **M6G** derived from it, are monomers in softwood lignification and are highly elevated in a CAD-deficient *Pinus taeda* mutant; whether the syringyl analogs are also produced and incorporated into angiosperms is not known. Arylglycerols **M7** are implicated by glycerol structures **X7** in lignins, but may come from the isolation process. Various acylated monolignols **M9–M11** are implicated in many plants. Tyramine hydroxycinnamate **M8**, particularly tyramine ferulate **M8G**, appears to be a monomer in tobacco lignins. Note that it is conventional to use α , β , and γ for the side chain positions in the hydroxycinnamyl alcohols **M1** and related products, but 7, 8, and 9 for the analogous positions in hydroxycinnamyl aldehydes **M2** and hydroxycinnamate esters **M4** and **M8**. Bracketed compounds have not been established as authentic monomers. (*Lignin Polymer Units*) Units are generally denoted based on the methoxyl substitution on the aromatic ring as **H**, **G**, **S** (and **5H**); dashed bonds represent other potential attachments via coupling reactions. The most common structures in lignins from normal and transgenic plants are shown as structures **A–L** with the bond formed during the radical coupling step (in bold); *p*-hydroxyphenyl units are not shown. The dashed bonds indicate substitutions by methoxyl (in syringyl components) or other attachments from coupling reactions; generic side chains are truncated (zigzag lines). Most units arise from cross-coupling reactions of a monomer with the growing polymer or by polymer-polymer coupling reactions. Resinol units **C** are from monolignol-monolignol coupling (followed by further cross-coupling reactions). Most 5–5-linked units **D** are in the form of dibenzodioxocins **D2**. *bis*-Aryl ether units **A2** are rare in most lignins, but relatively prevalent in tobacco. Units **A3** are seen in isolated lignins but may result from the isolation process. Units **F**, β -1 structures, may not occur in lignins as drawn but as spirodienones, for example (129). Benzodioxanes **J** are the main units resulting from the incorporation of 5-hydroxyconiferyl alcohol **M15H** monomers into lignins, particularly in COMT-deficient angiosperms. Units **K** are prevalent in CAD-deficient angiosperms and arise from endwise coupling of hydroxycinnamyl aldehydes **M2** into lignins. Unit **L** is a single example of a ferulate-monolignol cross-coupling product seen in grass lignins. (*End Groups*) End groups arise from coupling reactions that are not at the sidechain β -position. Hydroxycinnamyl end groups **X1** arise from dimerization reactions and represent only a small percentage of the units (2). End groups **X2** to **X6** derive from the corresponding monomers **M2–M6**; **X6b** is possibly an isolation artifact from oxidation of **X6** units. Glycerols **X7** may be from monomers **M7** or may be produced during ball milling from β -ether units **A**. (*γ -Acylated Units*) Any of the units **A–L** bearing a γ -OH may also bear an acyl group, partial structures **Y1–Y3**. They arise almost certainly from the corresponding monolignols **M9–M11**. (*Miscellaneous*) Finally, some other groups resulting from incorporation reactions are not accommodated by the other structures. Partial structures **Z1** arise from incorporation of monomer **M8**; general aldehydes can arise from hydroxycinnamyl aldehyde monomers **M2**; general esters result from the incorporation of ferulates and dehydrodiferulates.



Figure 4 Stem xylem phenotype of transgenic poplar downregulated for CAD. Debarked stems of 4-year-old, field-grown wild type (*left*) and transgenic poplar with reduced CAD activity (*right*), displaying the bright-red xylem coloration typical of plants with reduced CAD activity. Note: Cut transverse ends have been marked with paint to identify the line; see inset for unpainted transverse section showing restriction of the red color to developing xylem (© Nature McMillan, reprinted with permission).

Poplar Milled Wood Lignin Models (Wild type, and COMT-deficient)

This document and the associated [web page](#) (from which 3D and other files may be obtained) accompany a review article for [Annual Reviews in Plant Biology](#) on “Lignin Biosynthesis” by Wout Boerjan, John Ralph, and Marie Baucher, 2003, 54:519-546. The structures were constructed based loosely on the following partial data from NMR spectra (the [HMQC experiment](#) of which is shown) described in the following references.

- Marita JM, Ralph J, Lapierre C, Jouanin L, Boerjan W. 2001. [NMR characterization of lignins from transgenic poplars with suppressed caffeic acid O-methyltransferase activity](#). J. Chem. Soc., Perkin Trans. 1: 2939-45.
- Ralph J, Lapierre C, Marita J, Kim H, Lu F, Hatfield RD, Ralph SA, Chapple C, Franke R, Hemm MR, Van Doorselaere J, Sederoff RR, O'Malley DM, Scott JT, MacKay JJ, Yahiaoui N, Boudet A-M, Pean M, Pilate G, Jouanin L, Boerjan W. 2001. [Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR](#). Phytochem. 57: 993-1003.

Lignin	A	B	C	D	J	X	4-O-5	S/G
Wild-Type	88	3	7	0	0	2	?	0.725
COMT-sense	53	13	5	6	18	5	?	0.125

(see the NMR Figure for the designations of the units, **A-D** etc – same as in Fig 2 of the review manuscript).

We chose to use 20 phenylpropanoid units (i.e. constructed from 20 monomers), and included a single 4-O-5 branchpoint in each. No β -1 structures are included (since these are not seen at any significant level in the milled wood lignins).

The unit breakdown is as follows (note the units do not add up to 20 because of the way linkages may involve more than one unit, but the total number of **S+G+5H** units is 20).

WT	linkage	S	G	5H	S-S	S-G	G-G	5H-G	5H-S	5H-5H
A	β -O-4				3	9	4			
B	β -5				-	-	1			
C	β - β				1	-	-			
D2	DBDOX				-	-	-			
J	Benzodioxane				-	-	-			
X1	CA-endgroup	-	-	1						
E	4-O-5				-	1	-			
Total No		10	10	0						

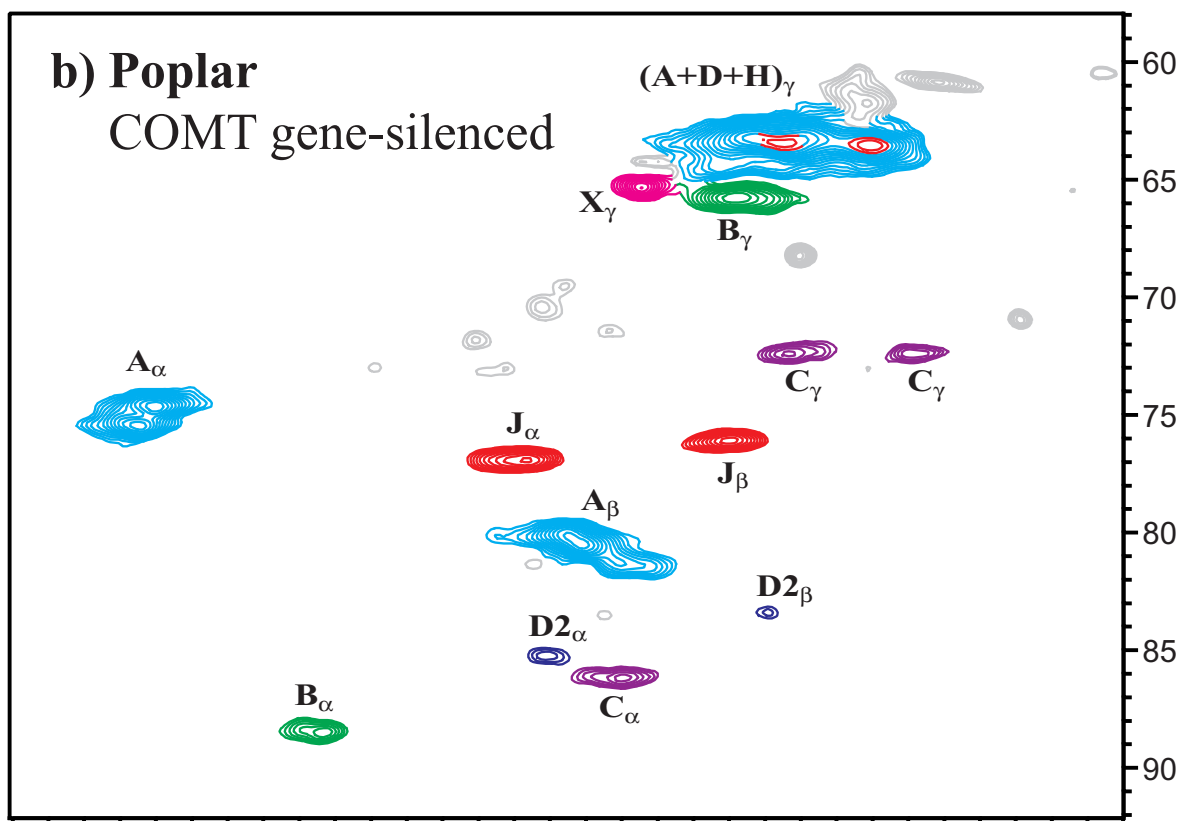
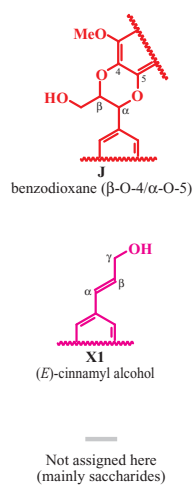
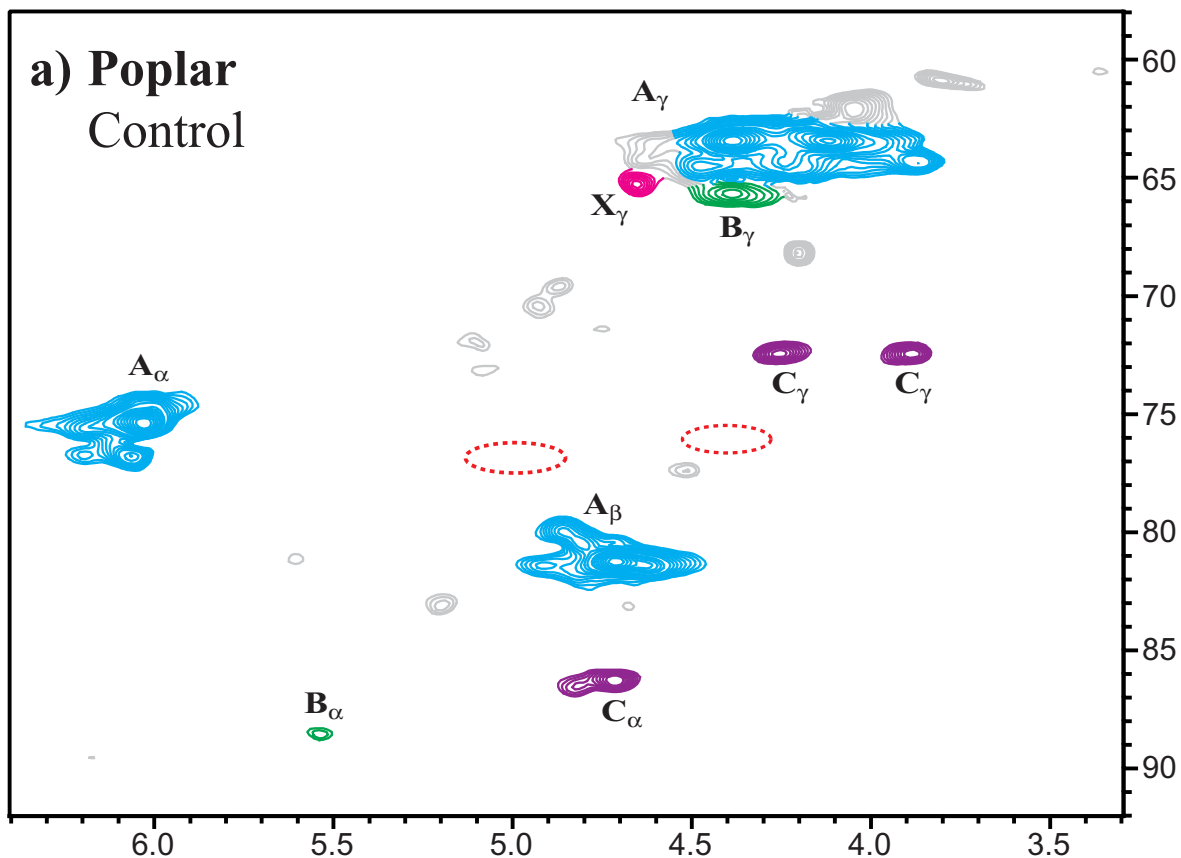
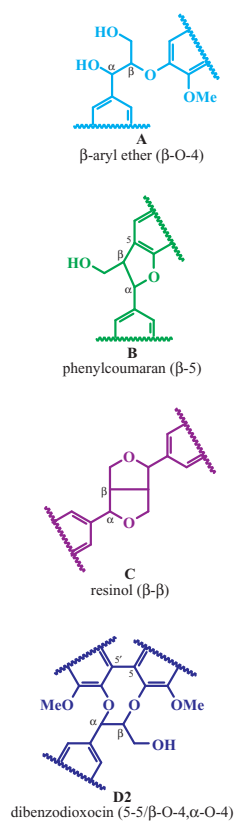
COMT-	linkage	S	G	5H	S-S	S-G	G-G	5H-G	5H-S	5H-5H
A	β -O-4					1	5	1	1	
B	β -5				-	-	3			
C	β - β					1	-			
D2	DBDOX				-	-	1			
J	Benzodioxane				-	-	-	3		1
X1	CA-endgroup	-	1	-						
X7	glycerol endgroup	-	-	1						
E	4-O-5				-	1	-			
Total No		2	14	4						

Notes

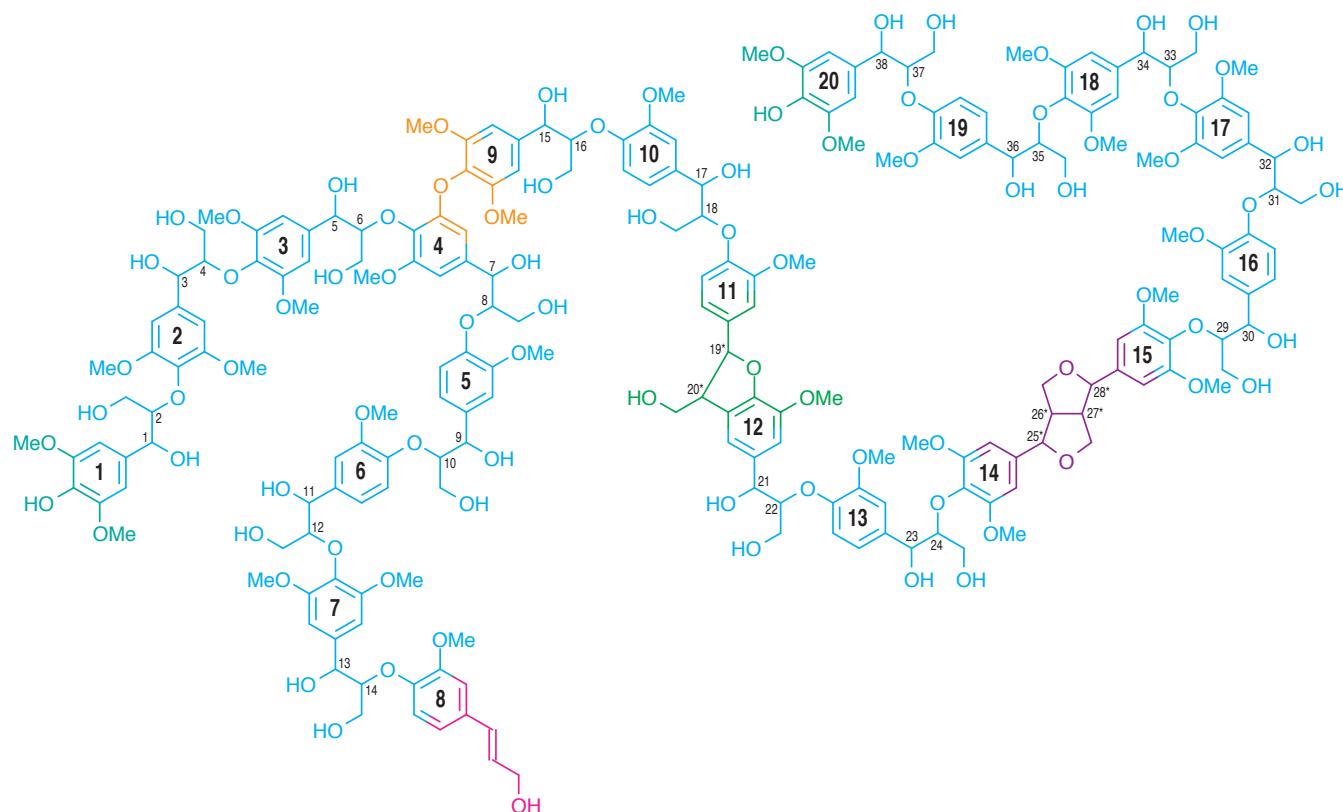
1. The colors of contours in the [NMR spectra](#) and of the groups in the [jpeg stills](#) and the 3D ViewerLite (.msv) files are similar. This way it is readily seen in the [NMR spectra](#) and in the models that, for example, the β -ether units **A** (cyan) are prevalent in the wild-type lignins, but less prevalent in the COMT-deficient lignin. Since it is only these units that cleave by thioacidolysis, DFRC, or in pulping reactions, it is clear why the COMT-deficient lignin is less easily degraded. The new benzodioxane units **J** in the COMT-deficient lignin are colored red (in the NMR and in the models). The branch points (4-O-5 units **E**, orange; dibenzodioxocin units **D2**, dark blue) are also easily identified in the Models.
2. The wild type has only a single branchpoint (4-O-5); the COMT-deficient has two (a 4-O-5 **E** again, and now a dibenzodioxocin **D2**, due to its higher guaiacyl content, as revealed by the NMR). Branchpoints provide the only deviation from “linear” structures.
3. The structures represent only *one* of many millions of isomers as described on the 2D structural pages, [page 3 for the wild type](#), and [page 4 for the COMT-deficient](#) lignins. For the wild type, we calculate that there are 2³³ (8,589,934,592) real viable isomers for the structure shown. For the COMT-deficient model we deduce 2²⁷ (134,217,728) physically distinct isomers. {This note is provided to emphasize the important point that their racemic nature alone confers incredible complexity and variability on polymeric lignins}.
4. The structures have been minimized in place, but it must be emphasized that each unit is probably just a local minimum – full optimization over all conformational space in a molecule of this size is impractical. They are ONLY MODELS!

Acknowledgements: These models are derived from data in the papers mentioned above. and based on [Brunow's softwood lignin model](#) (in Biopolymers. 2001. vol 1, pp 89-116). In addition, special thanks to Jane Marita for determining this data, to Tom Elder (Auburn University) and Ray Fort (U. Maine) for discussion and for alerting us to the Accelrys ViewLite program – the only one we currently know of that will allow us to color-code the lignin units, - and to Bill Lindmeier for arranging the web page.

HSQC NMR Spectra



2D Model Structure: Wild-type Poplar Milled Wood Lignin

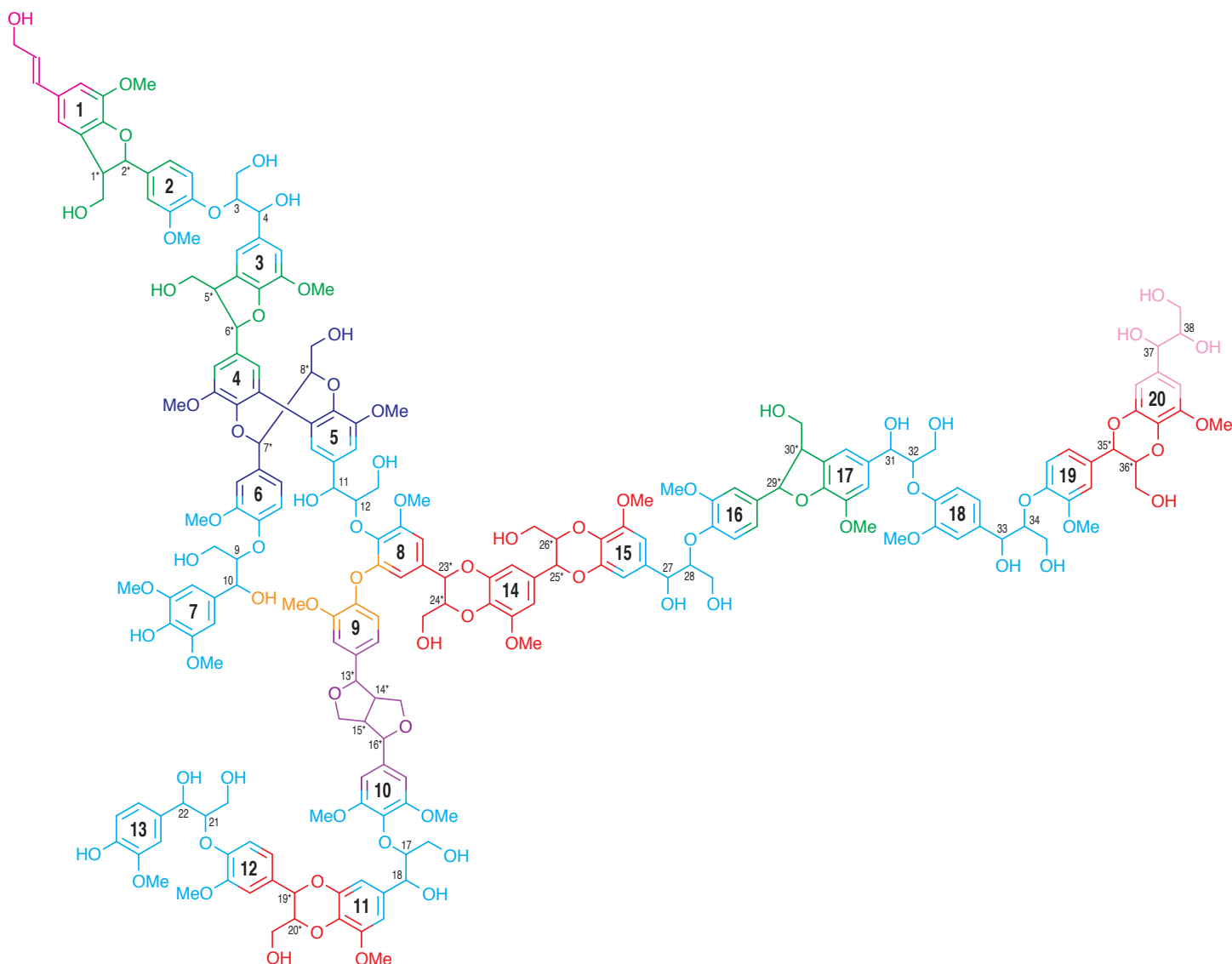


For the model for milled wood lignin from the wild type poplar, containing 20 phenylpropanoid units, there are 38 optical centers leading to 2^{38} potential optical isomers. However, the relative stereochemistries of 3 pairs of centers are fixed (asterisked centers; e.g. trans-ring junctions in phenylcoumarans). We therefore insinuate that there are 2^{35} optical isomers and therefore half that number of physically distinct isomers, i.e. 2^{34} (17,179,869,184) real viable isomers for the structure shown.

Pictures of the 3D Model are on [page 5](#).

The .pdb file for viewing in any molecular display program, e.g. RasMol and the .msv file for using with Accelrys' ViewerLite (with the same color scheme as used here to identify the lignin subunits) are available from our web site at <http://www.dfrc.ars.usda.gov/LigninModels.html>

2D Model Structure: COMT-deficient Poplar Milled Wood Lignin

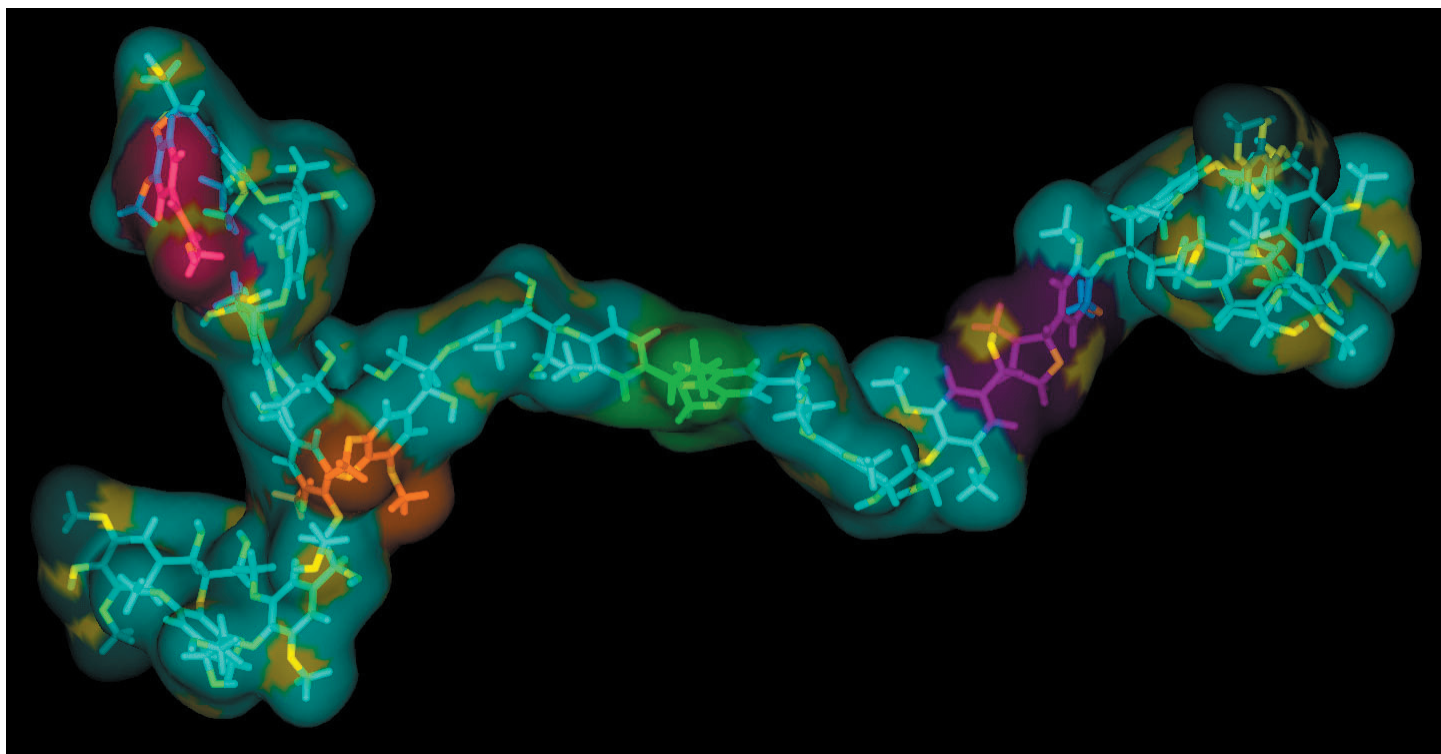
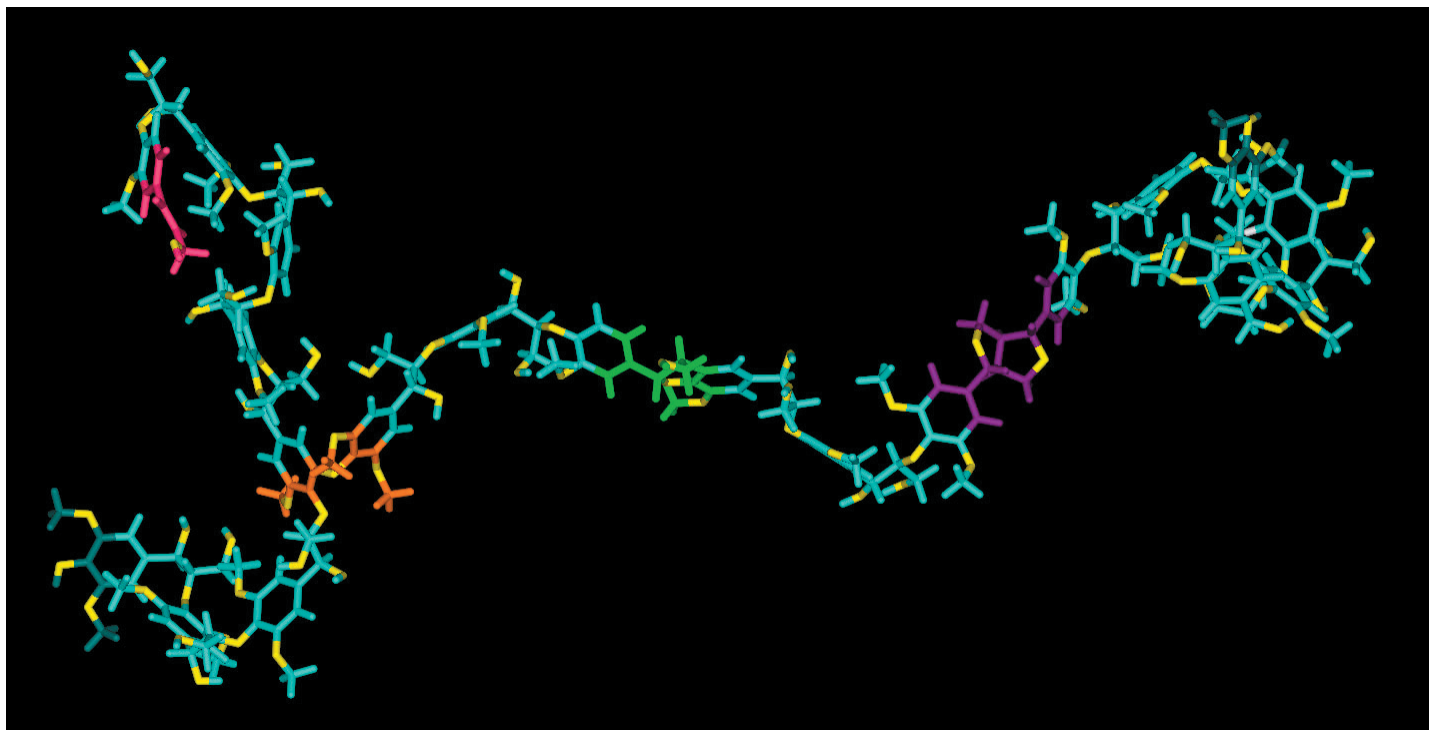


For the model for milled wood lignin from the COMT-deficient poplar, also containing 20 phenylpropanoid units, there are again 38 optical centers leading to 2^{38} potential optical isomers. However, the relative stereochemistries of 10 pairs of centers are fixed (asterisked centers; e.g. *trans*-ring junctions in phenylcoumarans, *trans*-ring junctures in the benodioxanes) in this case; the significant incorporation of 5-hydroxyconiferyl alcohol leads to a high proportion of benzodioxane structures which are mostly in the *trans*-ring form. We therefore calculate that there are 2^{28} optical isomers and therefore half that number of physically distinct isomers, i.e. 2^{27} (134,217,728) real viable isomers for the structure shown.

Pictures of the 3D Model are on [page 6](#).

The .pdb file for viewing in any molecular display program, e.g. RasMol and the .msv file for using with Accelrys' ViewerLite (with the same color scheme as used here to identify the lignin subunits) are available from our web site at <http://www.dfrc.ars.usda.gov/LigninModels.html>

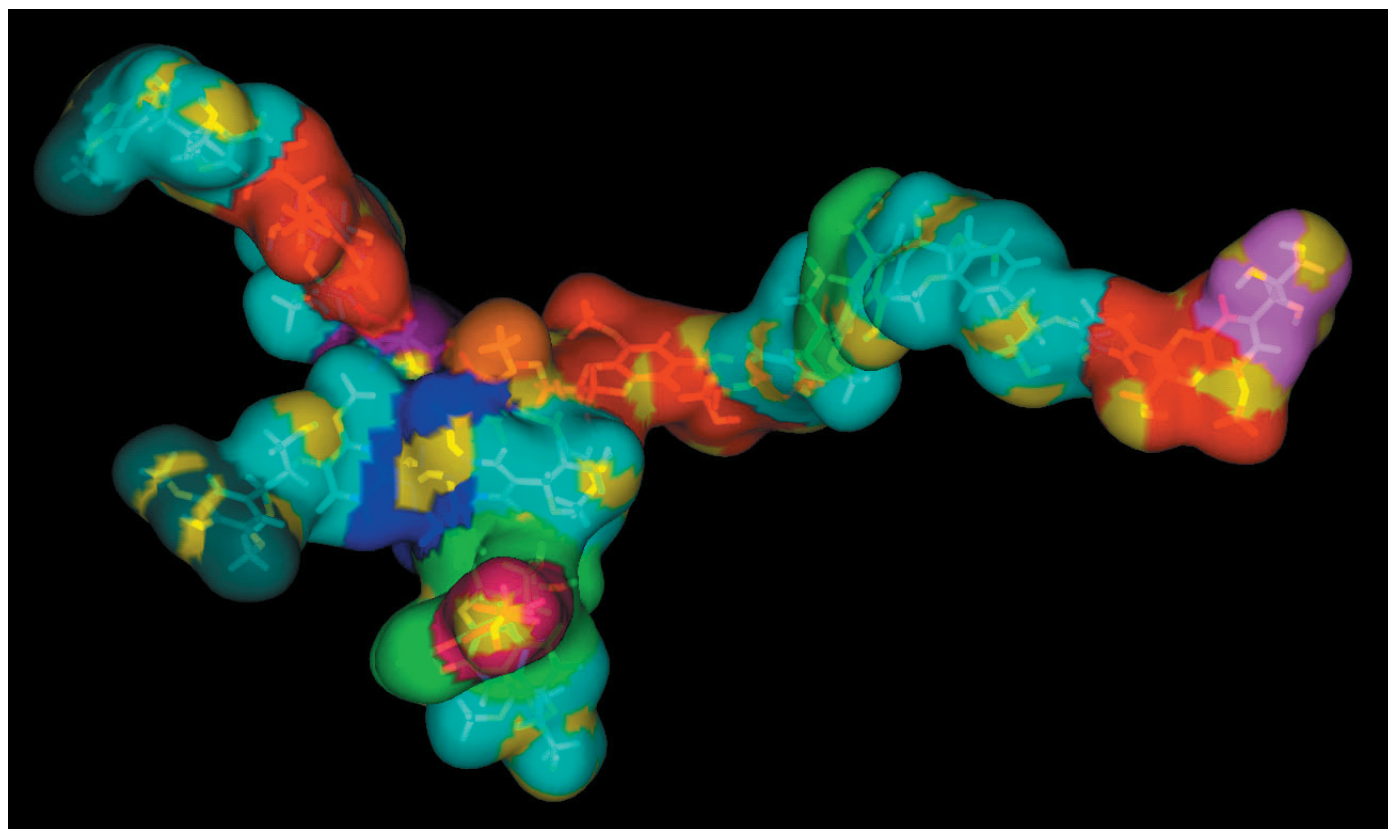
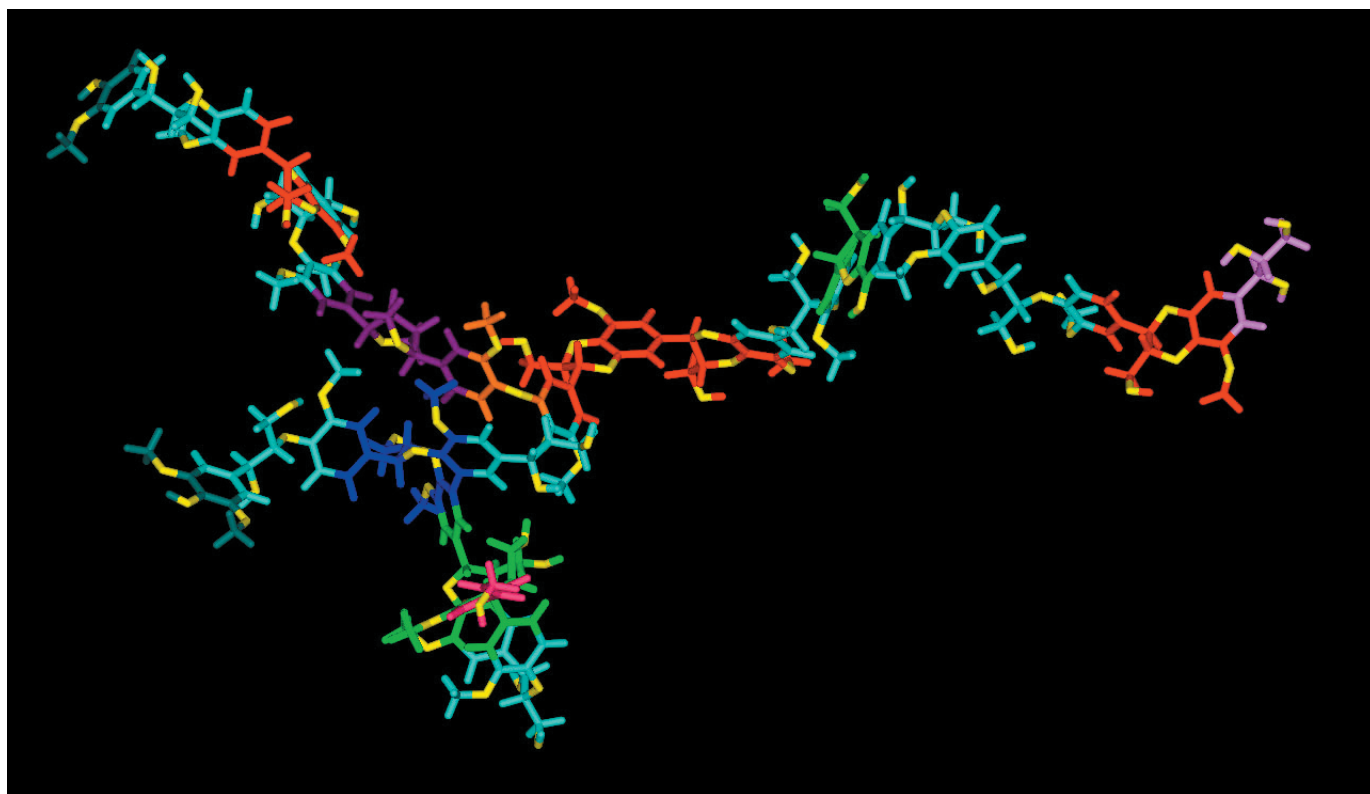
3D Model Structure: Wild-type Poplar Milled Wood Lignin



Stills are from the ViewerLite program.

The .pdb file for viewing in any molecular display program, e.g. RasMol and the .msv file for using with Accelrys' ViewerLite (with the same color scheme as used here to identify the lignin subunits) are available from our web site at <http://www.dfrc.ars.usda.gov/LigninModels.html>

3D Model Structure: COMT-deficient Poplar Milled Wood Lignin



Stills are from the ViewerLite program.

The .pdb file for viewing in any molecular display program, e.g. RasMol and the .msv file for using with Accelrys' ViewerLite (with the same color scheme as used here to identify the lignin subunits) are available from our web site at <http://www.dfrc.ars.usda.gov/LigninModels.html>